Escherichia coli isolated from feces of brown bears *(Ursus arctos)* have a lower prevalence of human extraintestinal pathogenic *E. coli* virulence-associated genes

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Abstract

Eighty-six *Escherichia coli* strains from feces of either wild brown bears or those living in a zoo were screened for phylogenetic groups using the revisited Clermont phylotyping method and the prevalence of 24 virulence-associated genes (VAGs) of extraintestinal pathogenic *E. coli* (ExPEC). Our results showed that most strains of *E. coli* in bears belonged to phylogenetic groups III/IV/V (29%) and B1 (26%). Only half of the tested VAGs were found in the *E. coli* bear strains, with *fimH* present in 72%, *ompT* in 63%, and *kpsMT* in 43% of the strains. When the data obtained on the fecal *E. coli* strains from brown bears were compared with the data obtained on 90 fecal *E. coli* strains from healthy humans, there were significant differences in *E. coli* population structures between both hosts.

Résumé

*Quatre-vingt-six souches d'*Escherichia coli *provenant de fèces d'ours brun vivant en nature ou dans un zoo ont été analysées pour déterminer les groupes phylogénétiques à l'aide de la méthode de phylotypage Clermont révisée et la prévalence de 24 gènes associés à la virulence (GAVs) d'*E. coli *pathogène extra-intestinal (ExPEC). Nos résultats ont montré que la plupart des souches d'*E. coli *chez les ours appartenaient aux groupes phylogénétiques III/IV/V (29 %) et B1 (26 %). Seulement la moitié des GAVs testés ont été trouvés dans les souches d'*E. coli *d'ours,* fimH *étant présent chez 72 %,* ompT *chez 63 %, et* kpsMT *chex 43 % de ces souches. Lorsque les résultats des souches d'*E. coli *obtenues des matières fécales d'ours brun ont été comparés aux données obtenues à partir de 90 souches fécales d'*E. coli *d'humains en santé, il n'y avait aucune différence significative dans la structure des populations d'*E. coli *entre les deux hôtes.*

(Traduit par Docteur Serge Messier)

Mammals have a complex gut microbiota that is shaped by intestinal anatomy, function, and diet (1). *Escherichia coli* is part of the normal intestinal microbiota and coexists with its host in mutual benefit. The intestinal microbiota can also be a reservoir of extraintestinal pathogenic *E. coli* (ExPEC) and it is known that animals, e.g., cats, dogs, and birds, can be a potential reservoir of ExPEC (2). Although extraintestinal infections due to *E. coli* are not known to be a significant issue in bears, some reports link *E. coli* with extraintestinal infections. For example, *E. coli* was isolated from cellulitis of an American black bear *(Ursus americanus)* (3) and *E. coli* septicemia was found in neonatal Polar bears *(Ursus maritimus)* (4).

The pathogenic potential of *E. coli* is closely related to the presence of virulence-associated genes (VAGs), such as adhesins, capsules, iron uptake systems, invasins, and toxins (5). While the brown bear *(Ursus arctos)* is the most widely distributed of bears, it is nevertheless an endangered species about which we lack data. As there are no data on *E. coli* strains from the intestinal microbiota of the brown bear, the aim of this study was to investigate and characterize fecal *E. coli* from brown bears for phylogenetic group and most typical ExPEC VAGs.

The phylogenetic group of the studied strains was determined using the revisited Clermont method, which is a novel quadruplex polymerase chain reaction (PCR) with a higher rate of correct classification (over 95%) than the traditional Clermont method (6). In addition, the sequence types (ST) of the B2 phylogenetic group strains were identified. Analysis with PCR was also used to determine the prevalence of VAGs among the studied *E. coli* strains. As composition of the intestinal microbiota is affected by the diet and environment of the host (7), we also compared *E. coli* strains from wild bears with bears housed in a zoo. Furthermore, the obtained data were compared with data gathered on 90 fecal *E. coli* strains from healthy humans to gain insight into host adaptation to *E. coli.*

We investigated a collection of 86 *E. coli* strains isolated from the feces of brown bears. Forty-five strains were isolated from the feces of wild brown bears located in the forest region of Kočevje, Slovenia and 41 strains were from 3 captive adult brown bears housed at the Ljubljana Zoo in Slovenia. The studied *E. coli* strains were isolated from 18 different samples of brown bear feces that were collected from October 2010 to April 2012. Four fecal samples were from brown bears in the zoo and 14 were from brown bears living in the

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Table I. Comparison of distribution of phylogenetic groups among fecal *E. coli* strains from brown bears and from humans determined using the revisited quadruplex Clermont method

Phylogenetic group	Prevalence (% of the tested strains) Bears (86 strains)	Humans (90 strains)	*P-value**
A	13	13	
B1	26	11	
B2	6	33	0.00003
C	0	1	
D	3	8	
E	15	21	
F	6	7	
I/II	1	0	
III/IV/V	29	2	3.3×10^{-6}
Unknown	1	3	

* *P*-values were calculated and only those that were statistically significant are given.

Table II. Comparison of prevalence of virulence-associated genes (VAGs) among fecal *E. coli* strains from brown bears and from humans

Virulence-associated genes (VAGs)	Prevalence [number (%) of the tested strains] Bears (86 strains)	Humans (90 strains)	*P-value**
Toxins			
cnf1	0	6	
hlyA	0	8	
usp	7	68	1.4×10^{-16}
ibeA	16	13	
Fimbriae and/or adhesins			
fimH	72	88	
papGII	0	8	
papGIII	0	3	
sfaDE	0	17	0.00081
afa/draBC	0	4	
Iron uptake			
iucD	2	39	7.9×10^{-9}
fyuA	21	66	4.7×10^{-8}
ireA	0	20	0.00008
iha	0	39	3.1×10^{-11}
hbp	0	8	
iroN	9	29	0.02606
Capsule			
kpsMT	43	58	
neuB	0	27	5.6×10^{-7}
Other			
tcpC	0	8	
APEC-*ompT*	2	22	0.00118
ompT	63	71	
clbAQ	0	16	0.00177
traJ	2	26	0.00013
iss	6	14	
traT	14	62	6.4×10^{-10}

* Only statistically significant *P*-values are given.

wild. The *E. coli* strains were initially isolated as lactose-positive colonies on MacConkey agar plates. Subsequently, growth was monitored on eosin methylene blue (EMB), as well as Uriselect plates and the indole test. Enterobacterial repetitive intergenic concensus-polymerase chain reaction (ERIC-PCR) was carried out to ascertain that only distinct *E. coli* strains were included in the study (8). The investigated strains were cultivated in Luria Bertani medium or agar.

Ninety *E. coli* strains isolated from the feces of healthy human volunteers, which were collected and partially characterized in a previous report (9), were also included in the present study. Bacterial deoxyribonucleic acid (DNA) used for PCR analyses was extracted according to standard protocols. All strains were assigned to phylogenetic groups A, B1, B2, C, D, E, F, I/II, III/IV/V, or unknown, using the revisited quadruplex Clermont method (6). The amplified DNA fragments were visualized by agarose gel electrophoresis using ethidium bromide staining. Amplicons were photographed with UV exposure and their lengths verified by a DNA ladder standard. The phylotyping was done in duplicates.

Escherichia coli bear strains assigned to the B2 phylogenetic group were additionally characterized by multilocus sequence typing (MLST), which was carried out as previously described by Wirth et al (10). Nucleotide sequences of genes *adk, fumC, gyrB, icd, mdh, purA,* and *recA* were amplified with primers and PCR conditions listed in Supplementary Table I and the primers and PCR conditions used to amplify VAGs are summarized in Supplementary Table II (both available at: http://www.bf.uni-lj.si/fileadmin/users/1/biologija/genetika/E-coli-from-brown-bears-Supplementary-Tables.pdf). Deoxyribonucleic acid (DNA) sequencing of the purified PCR products was conducted by Macrogen (South Korea). The obtained sequences were compared with known alleles and sequence types (STs) were assigned using MLST database for *E. coli* (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

The 24 analyzed ExPEC VAGs were: *ompT* (outer membrane protease T); APEC-*ompT* (avian pathogenic *E. coli* outer membrane protease T); *clbAQ* (genomic island that encodes the genotoxin colibactin); *traJ* (positive regulator of conjugation); *iss* (increased serum survival); *traT* (serum resistance); *neuB* (capsule K1, sialic acid Neu5Ac synthase); *kpsMT* (group II capsule); *papGII* (pyelonephritis-associated adhesin gene class II); *papGIII* (pyelonephritis-associated adhesin gene class III); *sfaDE* (S fimbrial adhesion); *afa/draBC* (adhesins of the AFA-DR family); *cnf1* (cytotoxic necrotizing factor); *hlyA* (hemolysin); *usp* (uropathogenic specific protein); *iucD, fyuA, ireA,* and *iroN* (siderophore receptors); *tcpC* (TIR homologous protein); *ibeA* (brain microvascular endothelial cells invasion); *fimH* (type 1 fimbriae); *iha* (iron-regulated gene A homologue adhesin); and *hbp* (hemoglobin protease).

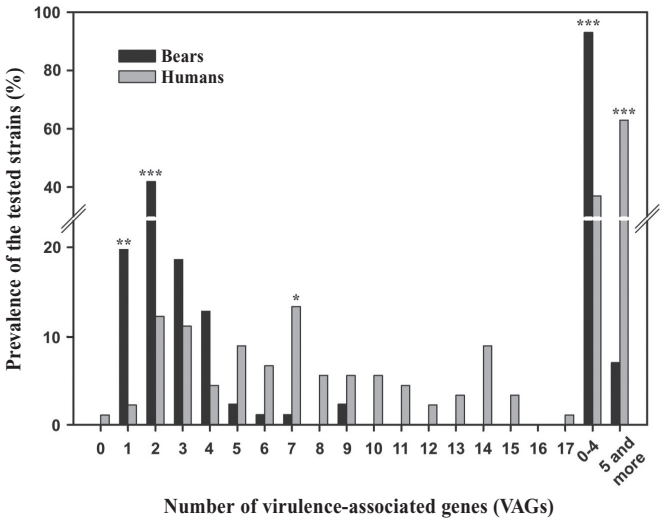

Figure 1. Comparison of VAG numbers among fecal *E. coli* strains from brown bears and human fecal *E. coli* strains. Only statistically significant *P*-values are given: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Polymerase chain reactions (PCR) were carried out in an automated thermal cycler (UNOII; Biometra, Göttingen, Germany) in 25 μL reaction mixtures containing 5 μL of template DNA, 20 pmol of forward and reverse primer, 0.2 mM 2′deoxynucleoside 5′-triphosphate (dNTP), 2.5 mM magnesium chloride ($MgCl_2$), 1× *Taq* buffer, 0.625 U *Taq* DNA polymerase, and distilled water to 25 μL (Fermentas, Vilnius, Lithuania). All the PCR reactions were done in duplicate. Prevalence of VAGs in strains from bears and healthy humans was analyzed using Fisher's exact test (2-tailed) (http://www.langsrud.com/fisher.htm). Due to multiple correlations, the Bonferroni correction was applied. The threshold for statistical significance after Bonferroni correction was set at *P*-values of < 0.05.

All 86 studied *E. coli* bear strains and 90 human strains were assigned to phylogenetic groups using the revisited Clermont phylotyping PCR method (6). The obtained data are presented in Table I. With the revisited Clermont phylotyping method, 59 (69%) of the studied brown bear strains could be assigned to a phylogenetic group (A to F), while the remaining 27 strains (31%) belonged to cryptic or unknown clades (I to V). The revisited Clermont method revealed that strains of the B1 phylogenetic group were the most prevalent, with 22 strains (26%), followed by group E with 13 strains (15%), and group A with 11 strains (13%). However, among the investigated human strains, the largest phylogenetic group was B2 with 30 strains (33%). The difference in the prevalence of B2 strains in brown bears and in humans was statistically significant. Furthermore, strains of the cryptic clades III/IV/V were significantly more prevalent in brown bears than in humans (Table I).

The 5 B2 strains were further analyzed for sequence types (STs) using MLST. Three strains belonged to ST174, 1 to ST1459, while 1 strain had a new combination of alleles. The strain with a new ST type had the following combination of alleles: ADK175 — FUMC356, a GYRB that most resembles GYRB301 with 459/460 matches (on the position 357 is a T, while in GYRB301 is a C), ICD304 — MDH242, a PURA that most resembles PURA282 with 477/478 matches (on the position 329 is a G, while in the PURA282 is a T),

Table III. Comparison of distribution of phylogenetic groups and virulence-associated genes (VAGs) among fecal *E. coli* strains from brown bears in zoo and in the wild

	Prevalence [number (%) of the tested strains]		
Trait	Zoo (41 strains)	In the wild (45 strains)	*P-value**
Phylogenetic group			
A	24	2	0.02606
B1	7	42	0.00191
B2	10	2	
C	0	0	
D	0	7	
E	17	13	
F	12	0	
I/II	2	0	
III/IV/V	27	31	
Unknown	0	2	
Virulence-associated genes (VAGs)			
Toxins			
cnf1	0	0	
hlyA	0	0	
usp	12	2	
ibeA	20	13	
Fimbriae and/or adhesins			
fimH	73	71	
papGII	0	0	
papGIII	0	0	
sfaDE	0	0	
afa/draBC	0	0	
Iron uptake			
iucD	0	4	
fyuA	29	13	
ireA	0	0	
iha	0	0	
hbp	0	0	
iroN	0	18	
Capsule			
kpsMT	44	42	
neuB	0	0	
Other			
tcpC	0	0	
APEC-*ompT*	0	4	
ompT	71	56	
clbAQ	0	0	
traJ	0	4	
iss	0	11	
traT	2	24	

* Only statistically significant *P*-values are given.

and a RECA that most resembles RECA130 with 508/510 matches (on the position 361 is a C, while in the RECA130 is a T, and on the position 365 is a C, while in the RECA130 is a T).

The studied *E. coli* strains were screened for the prevalence of 24 VAGs commonly found among ExPEC strains (5). The obtained data are summarized in Table II. Among *E. coli* strains isolated from brown bears, 12 different VAGs were detected. The most prevalent were *fimH* in 62 strains (72%), *ompT* in 54 (63%), *kpsMT* in 37 (43%), *fyuA* in 18 (21%), and *ibeA* in 14 strains (16%). While the presence of all tested VAGs was ascertained among the *E. coli* strains isolated from humans, the 5 most prevalent were *fimH* (88%), *ompT* (71%), *usp* (68%), *fyuA* (66%), and *traT* (62%).

Comparison of VAG prevalence of *E. coli* strains between brown bears and humans showed that 12 out of 24 VAGs (*usp, sfaDE, iucD, fyuA, ireA, iha, iroN, neuB,* APEC-*ompT, clbAQ, traJ,* and *traT*) were found to be statistically significant less often among *E. coli* strains from brown bears than among those from healthy humans (Table II). Analysis of the number of VAGs present in fecal bear and human *E. coli* revealed that there were fewer statistically significant strains with 5 or more VAGs in bear strains than in human strains (Figure 1). This difference was also reflected in a lower average virulence score among brown bear strains than human fecal *E. coli* strains, 2.6 *versus* 7.2, respectively (Figure 1).

Comparison of *E. coli* strains obtained from brown bears in the wild and those living in a zoo showed that strains belonging to the phylogenetic group A were statistically significantly associated with captive bears and group B1 strains were associated with wild bears ($P < 0.05$). No major difference was detected in the prevalence of the tested VAGs in both bear populations (Table III).

A number of studies have explored the characteristics of *E. coli* strains from different animal hosts and their relevance for virulence potential (2). While previous studies have defined the fecal microbiota and intestinal metabolic activity of polar and grizzly bears (7,11,12), to our knowledge there is no data on molecular characterization of *E. coli* strains from the endangered brown bear. In the present study, we therefore focused on the phylogenetic distribution and ExPEC VAGs of *E. coli* strains from captive and free-roaming brown bears. Due to genetic diversity, *E. coli* strains may be assigned to phylogenetic groups. The first reliable PCR method enabled assignment to 1 of 4 phylo-groups: A, B1, B2, or D (13). In 2013, a new quadruplex PCR method was introduced (6) that recognized groups A, B1, B2, C, D, E, F, and cryptic clades I to V. Only the new method was used in our study, as it was identified as being more reliable (6).

In their study of predominant aerobic microbiota of black bears *(Ursus americanus)* and grizzly bears *(Ursus arctos),* Goatcher et al (14) showed that the microorganisms isolated from bear samples could also be found in plant, water, and soil samples from the study area. This indicates that the predominant bear microbiota were influenced by the foraging habits and surrounding environments of the bears. Brown bears consume a predominantly vegetative diet (15), although they can also consume meat and dairy products. Hunters may provide bears with carcasses of wild animals at feeding stations in their natural environment. Bears can also come in contact with human food as they approach villages that are close to woods, orchards, and farmlands and by rummaging through garbage. As bears have an excellent sense of smell, they also easily find food discarded by humans visiting the woods. Compared to bears in the wild, brown bears living in captivity are in closer contact with humans through caretakers who clean their cages and handle their food as well as visitors who sometimes throw food. The bears' drinking water can also be a source of bacteria. Bears in the wild generally use several water sources, while a natural pond in their enclosure is the only source of water for bears at the Ljubljana Zoo.

The differences in food and water supplies among bears living in the wild and those in captivity could affect the prevalence of *E. coli* phylogenetic groups. In general, in this study, the phylogenetic distribution of strains from wild and captive bears was similar, except that group A was statistically significantly associated with captive bears and group B1 was associated with wild bears ($P < 0.05$). These results correlate with the known fact that group A strains are more prevalent in carnivores and omnivores than in herbivores, while in herbivores, most *E. coli* strains belong to group B1 (16). Of the 5 B2 strains, 4 were isolated from captive bears. This is not unexpected as there is closer contact between bears and humans at a zoo. Even though group B2 strains are associated with human extraintestinal *E. coli* infections, they are also prevalent among human fecal microbiota (17). Multilocus sequence typing (MLST) analysis of the studied B2 strains in bears showed that 3 strains belonged to ST174. This ST was recently determined for a B2 porcine fecal strain with resistance genes against 7 antibiotics (amikacin, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin, cotrimoxazole, and tetracycline) (18), which indicates a high potential for adapting to different environments.

To further our understanding of *E. coli* strains from brown bears, we screened our strain collection for the prevalence of several extraintestinal VAGs. A high prevalence of VAGs *fimH, ompT, kpsMT,* and *fyuA* was detected, which again indicates a virulence potential, albeit low, among the examined *E. coli* strains from captive and wild brown bears.

The gathered data on phylogenetic groups and VAG prevalences among the studied bear fecal *E. coli* strains were compared to data on phylogenetic groups and VAG prevalences among human fecal *E. coli* strains. A significant difference was revealed in the phylogenetic structure of both *E. coli* populations. Among the brown bear fecal *E. coli* strains, there were less statistically significant B2 strains and more III/IV/V strains than in human fecal *E. coli* strains. A lower prevalence of VAGs was revealed in bear strains compared to human strains. Bear strains also have a lower number of VAGs per strain than in human strains. The obtained data clearly showed a significant difference in the genetic structure of the *E. coli* from the 2 hosts, bears and humans.

Virulence factors encoded in VAGs associated with ExPEC could represent fitness factors involved in intestinal colonization and survival among the B2 group strains (19). Ursids are characterized by a simple gut physiology with rapid digestion (1) that could eliminate the need for fitness factors and explain the low prevalence of the B2 group, as well as a general low prevalence of VAGs among the examined *E. coli* isolates from brown bears. Iron uptake systems, such as siderophores, are a good example of the fitness factor nature of the virulence factors encoded in the VAGs, as iron is essential for bacterial growth. In this study, we examined

the prevalence of 6 VAGs involved in iron acquisition. All 6 of the tested iron-acquisition systems were statistically significantly linked to human strains, while bear strains harbored only *fyuA* [prevalence of 18 (21%)], *iroN* [prevalence of 8 (9%)], and *iucD* [prevalence of 2 (2%)]. As this may be due to the different gut complexities of different hosts, our results support the idea that prevalence of different VAGs may reflect adaptation to commensal lifestyle in different hosts and ExPEC strains are only a by-product of commensalism (20). Further characterization of *E. coli* bear strains will contribute to a deeper understanding of the complex dynamics between *E. coli* and its host.

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